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Wezel, Gilles P. van; Takano, Eriko; Vijgenboom, Erik; Bosch, Leendert; Bibb, Mervyn J.

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The *tuf3* gene of *Streptomyces coelicolor* A3(2) encodes an inessential elongation factor Tu that is apparently subject to positive stringent control

Gilles P. van Wezel,[1,2] Eriko Takano,[1] Erik Vijgenboom,[2] Leendert Bosch[2] and Mervyn J. Bibb[1]

Author for correspondence: Gilles P. van Wezel. Tel: +44 1603 452571. Fax: +44 1603 456844. e-mail: gilles.vanwezel@bbsrc.ac.uk

[1] Department of Genetics, John Innes Centre, Colney, Norwich NR4 7UH, UK

[2] Department of Biochemistry, Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300RA Leiden, The Netherlands

In *Streptomyces coelicolor* A3(2), two genes, *tuf1* and *tuf3*, encode the apparent polypeptide chain elongation factors EF-Tu1 and EF-Tu3, respectively. While *tuf1* appears to code for the major EF-Tu, the function of *tuf3* is unknown. To assess the role of EF-Tu3, *tuf3* was subjected to mutational and transcriptional analyses. Replacement of the 5′-half of *tuf3* by an antibiotic resistance cassette had no detectable effect on phenotype, indicating that *tuf3* is not essential for growth or differentiation. The transcription start site of *tuf3* was located approximately 195 nt upstream of the translation start site. The sequence of the *tuf3* promoter (P_{tuf3}) resembles the consensus for the major class of eubacterial promoters, and P_{tuf3} was recognized preferentially by an RNA polymerase fraction enriched in σ^{hrdB}, the principal σ factor of *S. coelicolor*. Nuclease S1 mapping failed to reveal *tuf3* transcripts during growth of *S. coelicolor* in liquid culture, consistent with the apparent absence of EF-Tu3 in total protein extracts of the same strain. However, *tuf3* transcription was observed in cultures of *S. coelicolor* M145 shortly after nutritional shiftdown (which resulted in the disappearance of *tuf1* transcripts) and after addition of serine hydroxamate, both of which induce the stringent response. Transcription of *tuf3* was also observed in transition-phase and stationary-phase cultures of *S. coelicolor* J1681, a strain deleted for *bldA* (which specifies a $tRNA_{Leu}$ for the rare leucine codon UUA). In all of these examples, transcription of *tuf3* followed the production of ppGpp, consistent with the hypothesis that *tuf3* is subject to positive stringent control.

Keywords: elongation factor Tu, *tuf3*, *Streptomyces coelicolor* A3(2), stringent control, ppGpp

INTRODUCTION

The polypeptide chain elongation factor Tu (EF-Tu), responsible for delivering amino-acyl tRNAs to the translating ribosome, is one of the most abundant proteins in micro-organisms: it can constitute up to 10% of total cell protein in rapidly growing *Escherichia coli* cells (van der Meide *et al.*, 1983). Two *tuf* genes, *tufA* and *tufB*, encode EF-Tu in *E. coli*, and are very similar in nucleotide sequence. The Gram-positive actinomycete *Streptomyces ramocissimus* produces the antibiotic kirromycin, which binds specifically to EF-Tu, and possesses three *tuf* genes, designated *tuf1*, *tuf2* and *tuf3*. EF-Tu1 and EF-Tu2 show 85% amino acid sequence identity, and both of them share about 65% amino acid sequence identity with EF-Tu3 (Vijgenboom *et al.*, 1994). While *tuf1* encodes the major elongation factor EF-Tu1, the roles of *tuf2* and *tuf3* are unknown: polyclonal antibodies raised against EF-Tu2 and EF-Tu3 that had been produced in *E. coli* failed to detect either protein in extracts of *S. ramocissimus* cultures grown under a variety of conditions (Vijgenboom *et al.*, 1994). While *tuf2* appears to be absent

Abbreviations: 2-dog, 2-deoxyglucose; EF-Tu, elongation factor Tu; SHX, serine hydroxamate; Spc, spectinomycin; Str, streptomycin; Thio, thiostrepton.

The GenBank accession number for the *tuf3* sequence reported in this paper is X77040.

from most *Streptomyces* species, *Streptomyces coelicolor* A3(2) and many other streptomycetes contain close homologues of both *tuf1* and *tuf3* (van Wezel, 1994). Like *tufA* of *E. coli*, *tuf1* of *S. coelicolor* is located in the *str* operon (van Wezel *et al.*, 1994a, 1995), and is well-separated from *tuf3* on the physical map of the *S. coelicolor* chromosome (van Wezel *et al.*, 1995). While EF-Tu1 is abundant in *S. coelicolor*, EF-Tu3 could not be detected (van Wezel *et al.*, 1994a).

The stringent response, which was first characterized as a rapid reduction in stable RNA synthesis upon amino acid starvation, is a pleiotropic response to the accumulation of uncharged tRNAs, and there is considerable evidence to suggest that the highly phosphorylated nucleotide ppGpp plays a key role in mediating stringent control (Cashel & Rudd, 1987). *E. coli tufA* and *tufB* are subject to negative stringent control in response to amino acid starvation, with enhanced ppGpp levels coinciding with severely reduced levels of transcription of both genes (Reeh *et al.*, 1976). *S. coelicolor* also undergoes the stringent response (Takano & Bibb, 1994), which can be elicited either by nutritional shiftdown or by addition of serine hydroxamate (SHX; a competitive inhibitor of seryl-tRNA synthetase), and is characterized by ppGpp synthesis and a rapid decrease in the level of transcription of rRNA genes (Strauch *et al.*, 1991). Although the role of ppGpp in mediating the growth rate control of gene expression remains controversial (Hernandez & Bremer, 1990, 1993; Gaal & Gourse, 1990), recent evidence has implicated the highly phosphorylated nucleotide in stationary phase gene expression in *E. coli* (Gentry *et al.*, 1993). Interestingly, antibiotic production in streptomycetes is generally limited to stationary phase, and mutants of a number of *Streptomyces* species that are deficient in ppGpp synthesis are also deficient in antibiotic production, leading to the suggestion that ppGpp might serve as a general trigger for antibiotic biosynthesis (Ochi, 1986, 1987; Takano & Bibb, 1994, and references therein). Furthermore, there is a reasonably good correlation in *S. coelicolor* between ppGpp synthesis and the transcription of antibiotic pathway-specific regulatory genes (Takano & Bibb, 1994).

In an attempt to determine the role of *tuf3* in *S. coelicolor*, we have inactivated the gene by replacing part of it with an antibiotic resistance determinant. The transcription start site of *tuf3* was identified by *in vitro* and *in vivo* analyses, and transcription of *tuf3* was monitored during growth in liquid culture and after induction of the stringent response, either by nutritional shiftdown or by addition of SHX. The results suggest that *tuf3* is an inessential gene that is subject to positive stringent control.

METHODS

Bacterial strains and plasmids. *S. coelicolor* strains M145 (prototrophic, SCP1$^-$ SCP2$^-$; Hopwood *et al.*, 1985), J1501 (*hisA1 uraA1 strA1* Pgl$^-$, SCP1$^-$ SCP2$^-$; Chater *et al.*, 1982), J1501Δ*glkA* (E. Vijgenboom, unpublished data) and J1681 (J1501Δ*bldA*; Leskiw *et al.*, 1993) were obtained from the John Innes Centre strain collection. *E. coli* JM101 (Messing *et al.*, 1981) was the host for pUC18 (Yanisch-Perron *et al.*, 1985) and constructs derived from it.

pISCT3-1, pISCT3-2 and pISCT3-U1 were made by cloning fragments of the *S. coelicolor tuf3*-containing plasmid pBSCT3-1 (van Wezel *et al.*, 1994a) via pUC18 into the *xylE*-based promoter-probe vector pIJ4083 (Clayton & Bibb, 1990). Plasmid inserts were: *Pvu*II–*Mlu*I (−500/+1700) in pISCT3-1, *Kpn*I–*Mlu*I (−260/+1700) in pISCT3-2 and *Pst*I–*Kpn*I (−1900/−5) in pISCT3-U1 (Fig. 2). In each construct, *tuf3* or its upstream region was inserted in pIJ4083 in the same orientation as *xylE* (Zukowski *et al.*, 1983). However, since the constructs pISCT3-1 and pISCT3-2 also contain 500 bp of sequence downstream of *tuf3*, these constructs were not used for promoter probing.

The strain used for *tuf3* inactivation was *S. coelicolor* J1501Δ*glkA*, and the gene replacement vector was pIJ2559, a 12·2 kb construct derived from pBR329 (Covarrubias & Bolivar, 1982) that is unable to replicate in *Streptomyces* since it lacks an appropriate origin of replication (Fig. 1). In this construct the *aadA* gene (Prentki & Krisch, 1984), which confers spectinomycin (Spc) and streptomycin (Str) resistance on both *E. coli* and *Streptomyces*, is flanked on one side by the −1900/−260 *tuf3* upstream region and on the other by the 3·6 kb fragment containing the second half of *tuf3* and 3 kb of downstream sequence. Thus a double recombination event will replace the −260/+600 segment of *tuf3* by *aadA*. Additional selectable markers on pIJ2559 are *tsr* [conferring thiostrepton (Thio) resistance in *Streptomyces*], cloned as a 1 kb *Bcl*I fragment from pIJ4083, and *glkA* encoding glucose kinase, which was available as a 1·3 kb *Pst*I fragment on the plasmid pIJ2423 (Angell *et al.*, 1994). J1501Δ*glkA* is resistant to 2-deoxyglucose (2-dog), but the presence of the pIJ2559-derived *glkA* renders this strain 2-dog-sensitive. Successful inactivation of *tuf3* by the required double crossover results in loss of both *tsr* and *glkA*, yielding a Thio^S 2-dog^R phenotype.

Culture conditions. Surface-grown cultures were cultivated on R2YE or on minimal medium (MM) agar plates, using glucose or mannitol as the carbon source (Hopwood *et al.*, 1985). Liquid cultures were grown in a minimal medium supplemented with Casamino acids (SMM; Takano *et al.*, 1992), or in yeast extract/malt extract medium (YEME) with 0·5% glycine (Hopwood *et al.*, 1985), and were inoculated at a density of 5×10^6 c.f.u. ml^{-1} and grown at 30 °C with vigorous shaking (300 r.p.m. min^{-1}). Reproducibly dispersed growth was obtained, with a doubling time of 2·2 h in SMM and 1·9 h in YEME. In SMM, rapid transition into stationary phase occurred approximately 14 h after inoculation (Strauch *et al.*, 1991). Nutritional shiftdown and treatment with 25 mM SHX were performed as described by Strauch *et al.* (1991).

Promoter-probe experiments. The *xylE* gene from *Pseudomonas putida* (Zukowski *et al.*, 1983) present in pIJ4083 (Clayton & Bibb, 1990) was used as a reporter gene for *in vivo* promoter activity. Transformants containing pISCT3-U1 were grown on R2YE and MM (Hopwood *et al.*, 1985) in the presence of 10 μg Thio ml^{-1} (a gift from Bristol-Meyers Squibb). Plates were sprayed with 0·5 M catechol after 1, 2, 3 and 4 d growth and the amount of catechol converted into yellow 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase was assessed visually.

***In vitro* transcription analysis.** RNA polymerase was isolated from a culture of *S. coelicolor* M145 in the transition between exponential growth and stationary phase, as described previously by Buttner & Brown (1985), and partially separated into different holoenzyme forms by Superose-6 FPLC. *In vitro* run-off

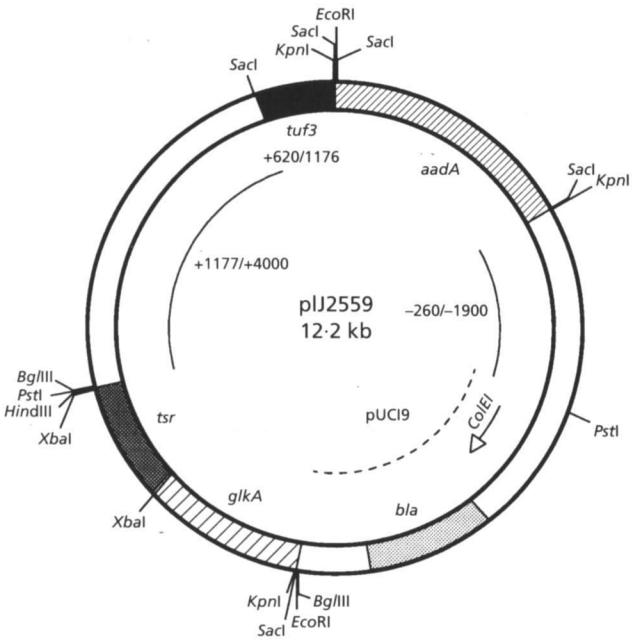

Fig. 1. **Restriction map of pIJ2559. Numbers refer to nt positions in the complete *tuf3* clone (pBSCT3-1), and are relative to the *tuf3* translation start site. ColE1, origin of replication E1 (from pBR322); *aadA*, Spc/Str resistance gene; *bla*, ampicillin resistance gene; *glkA*, glucose kinase gene; *tsr*, Thio resistance gene.**

transcription experiments were performed as described by Buttner & Brown (1985). Products were analysed on denaturing 6% (w/v) polyacrylamide gels using ^{32}P-end-labelled *Hpa*II fragments of pBR322 as size markers. The 600 bp *Pvu*II–*Sty*I (−500/+100) fragment and the 360 bp *Kpn*I–*Sty*I (−260/+100) fragment were isolated from pISCT3-1 and pISCT3-2, respectively (Fig. 2), and used as templates.

RNA isolation. RNA was isolated from M145, J1501 or J1681 according to Hopwood *et al.* (1985). To remove residual DNA, the RNA was salt-precipitated in 3 M NaOAc (pH 6·0). The RNA samples were then treated with DNaseI (1 h at 37 °C with 0·1 U DNaseI per 50 ml initial culture sample), extracted with a 1:1 mixture of phenol/chloroform (saturated with 100 mM Tris, pH 7·0) and precipitated in 0·4 M NaOAc (pH 6·0) with 2-propanol. The RNA was resuspended in water and the concentration was determined spectrophotometrically.

Nuclease S1 mapping. Hybridization of 10 μg RNA with the appropriate DNA probe was performed according to Murray (1986) in NaTCA buffer (Summerton *et al.*, 1983). All subsequent steps were carried out as described previously by Strauch *et al.* (1991), using an excess of probe. The 600 bp *Pvu*II–*Sty*I fragment from pISCT3-1 (Fig. 2), ^{32}P-labelled at the 5′ end of the *Sty*I site, was used for mapping *tuf3* transcripts; the 25 bp non-homologous extension upstream of the *Pvu*II site allowed discrimination between DNA–RNA hybrids and reannealed probe. The 530 bp *Sma*I–*Xho*I (−70/+460) fragment from pASCT1-1 (van Wezel *et al.*, 1994a; Fig. 2), ^{32}P-labelled at the 5′ end of the *Xho*I site, was used for mapping *tuf1* transcripts. The 558 bp *Fsp*I–*Hin*cII fragment, corresponding to nt positions −708 to −151 with respect to the 5′ end of the 16S rRNA coding sequence and ^{32}P-labelled at the 5′ end of the *Hin*cII site, was used for mapping *rrnA* transcripts (van Wezel *et al.*, 1994b).

ppGpp measurements. ppGpp levels were determined as described by Strauch *et al.* (1991).

RESULTS

Inactivation of *tuf3* by gene replacement

To study the role of *tuf3* in *S. coelicolor* we inactivated the gene by replacing the segment of *tuf3* that extends from −260 to +600 (with respect to the translation start site of

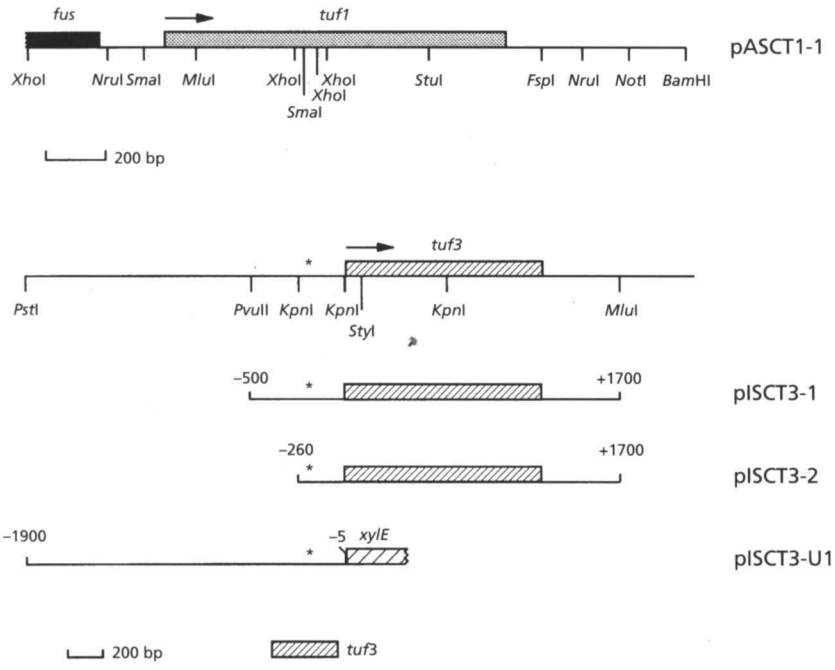

Fig. 2. ***S. coelicolor tuf*** **constructs. Only the plasmid inserts are shown. *fus* (which encodes EF-G) and *tuf1* are part of the S12 (*str*) operon, and were cloned in pAT153 (Twigg & Sherratt, 1980) to give pASCT1-1 (van Wezel *et al.*, 1994a); only part of the insert is shown. The *tuf3* constructs were made by inserting fragments into the multiple cloning site of pIJ4083. Only pISCT3-U1 was used for promoter-probing. Numbers above inserts correspond to nt positions relative to the translation start site of *tuf3*. Arrows show the direction of transcription. *, Transcription start site of *tuf3*.**

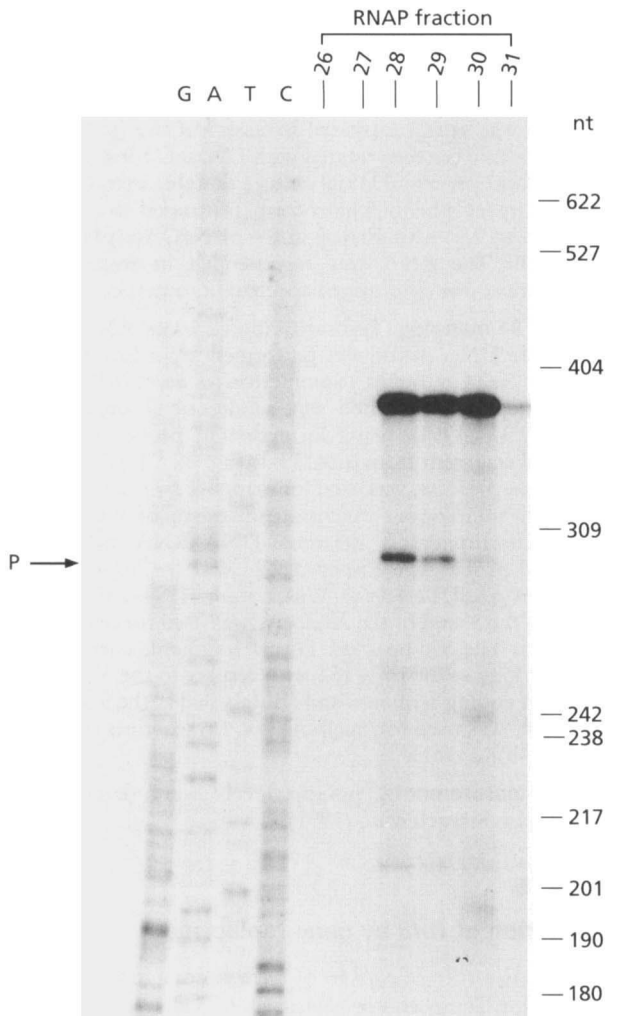

Fig. 3. *In vitro* transcription analysis of *S. coelicolor tuf3*. P, Run-off transcript originating at P_{tuf3}; RNAP fraction, RNA polymerase fractions eluting from a Superose-6 FPLC column. The right-hand side of the figure shows a DNA size marker (denatured *Hpa*II-digested pBR322) in nt. GATC, *tuf3* nt sequence ladder generated using a 19 nt primer whose 5′ end corresponds precisely to the 5′ end of the labelled probe used for S1 nuclease mapping (see Methods).

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-267   GGTACCGCTCTCGAACGGCCGTTCCATAAAAAACCATTCGACGTGCGACG
                                  ...
-217   AAGCGTCGGCGATGATCTGTCTCATGTTCCGGTACGCCTTCCACCTCGCA
-167   GCATCCGCGGTCGCGGATGCCCCGAAGGCTGCCGTCGCCCACTTCACCGC
-117   CGCAGTCGACGGCGCCCGAAGCTGACCCTCCCCGGATCGTCCGGCGGACC
 -67   CCGCAGGGGGAGGGTCGGCCGGCCCCAGGGGTCCCCACCACCGCCCACGA
            SD
 -17   GGCTTCGAGGTACCGCCATGTCCAAGACGGCGTACGTCCGCACCAAACCG
                      M  S  K  T  A  Y  V  R  T  K  P
```

Fig. 4. Nucleotide sequence of the region upstream of the *tuf3* structural gene. Numbering refers to nt position relative to the *tuf3* translation start site (chosen as +1). Putative promoter consensus sequences are underlined. Dots above the sequence refer to alternative transcription start sites of *tuf3*. An 8 nt A+T-rich stretch preceding the promoter is shown in bold typeface. The deduced amino acid sequence at the start of EF-Tu3 is shown below the sequence; the putative Shine–Dalgarno sequence (SD) preceding *tuf3* is represented in italics.

tuf3) with an antibiotic resistance gene (*aad*) that confers resistance to Spc and Str. This resulted in the deletion of the *tuf3* promoter (see later) and ribosome-binding site, and of protein-coding sequences essential for EF-TU function (van Wezel *et al.*, 1994a). Protoplasts of *S. coelicolor* J1501Δ*glkA* (*glkA* encodes glucose kinase which confers sensitivity to 2-dog) were transformed with the *tuf3* disruption construct pIJ2559 (see Methods and Fig. 1), selecting for resistance to Thio, Spc and Str. Resistant colonies were likely to have arisen from single-crossover integration of the plasmid into the *tuf3* region of the chromosome. Spores from five of the 20 independent pIJ2559 transformants obtained were plated on MM containing 2-dog and Spc to select for the second crossover event, which removed *glkA* and *tsr*, and resulted in replacement of the 5′ half of *tuf3* by *aadA*. Five putative *tuf3* mutants were screened by three Southern hybridizations, with probes recognizing *aadA* (Fig. 1), the N-terminal part of *tuf3* that should have been deleted, and the part of *tuf3* that should have remained. All five isolates did indeed lack the 5′-half of the *tuf3* gene and gave the expected hybridization patterns with all three probes. Since they also had all of the expected genetic markers (resistant to 2-dog and Spc, sensitive to Thio and unable to grow on glucose as a carbon source), they were considered to be *tuf3* deletion mutants. The mutants were indistinguishable from the parental strain J1501Δ*glkA* in their growth rates, colony morphology and ability to produce the pigmented secondary metabolites actinorhodin and undecylprodigiosin, and no morphological abnormalities could be detected at the microscopic level.

Expression of the *tuf3* promoter occurs in surface-grown cultures when present on a high-copy-number plasmid

M145 transformants containing the multi-copy plasmid pISCT3-U1, which has the −1900/−5 region (relative to the translation start site) of *tuf3* in front of *xylE* (Fig. 2), gave colonies that rapidly became yellow when sprayed with catechol after 2 d growth on solid MM containing mannitol as carbon source. After 4 d, spraying with catechol produced bright-yellow aerial hyphae. This suggests that catechol dioxygenase production (i.e. *xylE* transcription) takes place in both vegetative and aerial mycelium, although the pigmentation of the aerial hyphae might have been the result of diffusion of the yellow compound from the vegetative mycelium (diffusion of the product of XylE activity, the yellow compound 2-hydroxymuconic semialdehyde, into the agar was also clearly apparent). Interestingly, pISCT3-U1 transformants produced neither aerial mycelium nor the pigmented antibiotics actinorhodin and undecylprodigiosin on MM with glucose or on R2YE plates, even

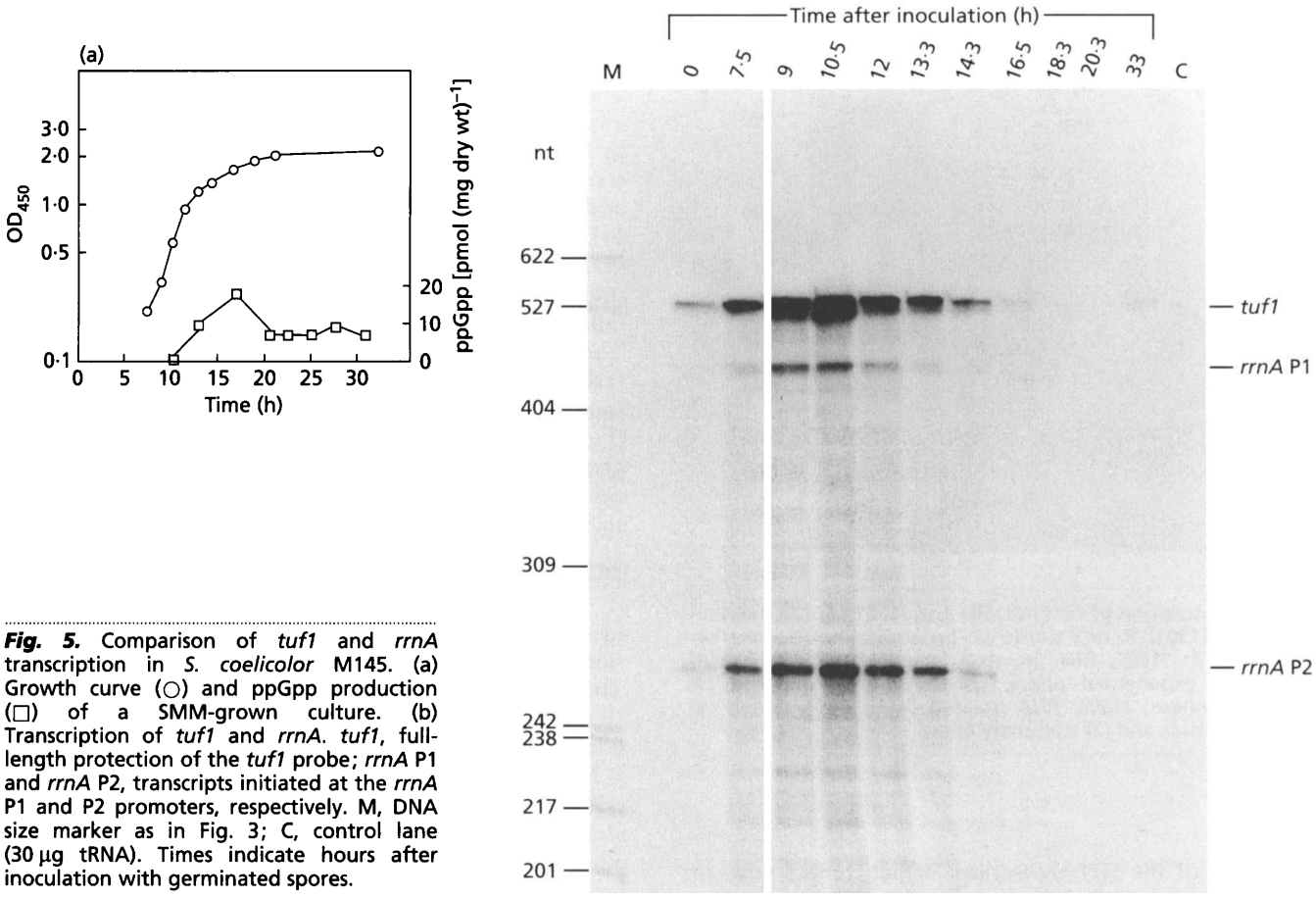

Fig. 5. Comparison of *tuf1* and *rrnA* transcription in *S. coelicolor* M145. (a) Growth curve (○) and ppGpp production (□) of a SMM-grown culture. (b) Transcription of *tuf1* and *rrnA*. *tuf1*, full-length protection of the *tuf1* probe; *rrnA* P1 and *rrnA* P2, transcripts initiated at the *rrnA* P1 and P2 promoters, respectively. M, DNA size marker as in Fig. 3; C, control lane (30 μg tRNA). Times indicate hours after inoculation with germinated spores.

after prolonged incubation at 30 °C, a phenotype comparable to that of an *S. coelicolor bldA* mutant (Lawlor *et al.*, 1987). M145 transformants containing pISCT3-1 or pISCT3-2 displayed normal growth and differentiation, regardless of the medium used.

In vitro transcription of *tuf3*

RNA polymerase from a transition phase culture of *S. coelicolor* M145, partially fractionated into different holoenzyme forms by Superose-6 FPLC, was used in *in vitro* run-off transcription assays to identify promoters in the *tuf3* upstream region. The *Pvu*II–*Sty*I (−500/+100) and the *Kpn*I–*Sty*I (−260/+100) fragments were used as templates. Since both fragments gave identical results, only those obtained with the *Kpn*I–*Sty*I template are shown (Fig. 3). In each case, two major transcripts were observed. One corresponded to the size of the template and was probably the result of end-to-end transcription. The second band (designated P in Fig. 3) corresponded to a transcript of approximately 295 nt and suggests a transcription start site around nt position −195 (with respect to the translation start site), which was confirmed by nuclease S1 mapping (see below). The putative transcription start site is preceded by the sequences TCGACG and GATGAT, separated by 17 bp (Fig. 4), which resemble the consensus −35 and −10 sequences, respectively, for the major class of eubacterial promoters.

In *S. coelicolor*, such sequences appear to be recognized by σ^{hrdB} (Brown *et al.*, 1992), whose activity was most abundant in RNA polymerase fraction 28 (data not shown), the one that gave the strongest signal for P_{tuf3} (Fig. 3).

Transcription of *tuf1* and *tuf3* during liquid culture

To establish the level and timing of *tuf3* transcription *in vivo*, RNA from M145 and J1501 cultures grown in SMM was analysed by nuclease S1 mapping using the 620 bp *Pvu*II–*Sty*I fragment as probe. Growth of, and ppGpp production by, the M145 culture used for RNA isolation are shown in Fig. 5(a). No protection of the *tuf3* probe was observed. We also analysed the transcription of *tuf1* in the *S. coelicolor* M145 culture, using the same RNA preparations. *tuf1* lies in the S12 ribosomal protein operon (van Wezel *et al.*, 1994a, 1995) and, by analogy to *E. coli* (Lindahl & Zengel, 1986, and references therein), is likely to be transcribed from a promoter upstream of *rpsL* (encoding S12). For mapping of *tuf1* transcripts we used the 530 bp *Sma*I–*Xho*I (−70/+460) fragment from pASCT1–1 (van Wezel *et al.*, 1994a), uniquely end-labelled at the *Xho*I site, as probe (Fig. 2), and therefore expected full-length protection of the probe. Since different probes were used, strict quantitative comparisons are not possible, but it is interesting to note that

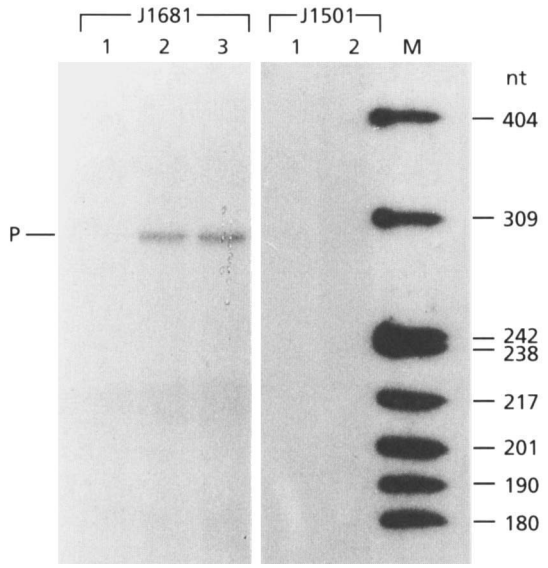

Fig. 6. Transcription of *tuf3* in J1501 and J1681 (a *bldA* deletion mutant of J1501). P, *tuf3* transcript. Lanes: M, DNA size marker as in Fig. 3; J1681, RNA isolated from J1681 (J1501Δ*bldA*) during (1) exponential phase, (2) transition phase and (3) stationary phase; J1501, RNA isolated from J1501 during (1) transition phase and (2) stationary phase.

the levels of the *tuf1* transcripts, which reflect rates of synthesis and degradation, were considerably higher than those derived from the *rrnA* P1 and P2 promoters (Fig. 5b), even though P2 is the strongest promoter of the *rrnA* operon (van Wezel *et al.*, 1994b). The control, 30 μg tRNA, failed to protect the probe, indicating that the full-length protection was not due to probe reannealing. Transcripts corresponding to *tuf1* and *rrnA* were barely detectable approximately 16 h after inoculation, corresponding to early stationary phase. Thus, transcription of *tuf1* shows the same growth-phase dependence as *rrnA*.

tuf3 transcription in the *bldA* deletion mutant J1681

Interestingly, nuclease S1 mapping using RNA from J1681, a *bldA* deletion mutant of J1501 defective in the formation of aerial mycelium and spores, showed *tuf3* transcripts in this strain (Fig. 6), but only after prolonged exposure of the autoradiograms. While no signal was detected during exponential phase (Fig. 6, J1681, lane 1), a band was detected in RNA isolated from late-exponential-phase and stationary-phase cultures. The 295 nt protected fragment corresponds in size to the transcript observed in the *in vitro* run-off transcription assays (Fig. 3). The transcription initiation site for the corresponding promoter is around nt position −195 relative to the *tuf3* translation start site. We failed to detect *tuf3* transcripts during transition phase in the congenic *bldA*$^+$ strain J1501 (Fig. 6, J1501, lanes 1 and 2, respectively), consistent with the data obtained for M145.

tuf3 is transcribed after nutritional shiftdown and after addition of SHX

Since transcription of *E. coli tufA* and *tufB* is negatively stringently controlled (Reeh *et al.*, 1976), we analysed how *tuf1* and *tuf3* transcription responded to amino acid starvation provoked by either nutritional shiftdown or by addition of SHX. Nutritional shiftdown of an *S. coelicolor* M145 culture at an OD_{450} of 0·6 led to a marked increase in the level of ppGpp, with a maximum of about 200 pmol (mg dry weight)$^{-1}$ 15 min after shiftdown (Fig. 7a), falling to 45 pmol (mg dry weight)$^{-1}$ 2 h after shiftdown. The culture continued growing (albeit at a slow rate) and reached stationary phase 6–7 h after shiftdown. *tuf1* transcripts were barely detectable 30 min after shiftdown (Fig. 7b), suggesting that expression of the major EF-Tu gene of *S. coelicolor* is also subject to negative stringent control. Reappearance of *tuf1* transcripts 4·8 h after amino acid depletion presumably reflects adaptation to the new growth rate and the turnover of excess EF-Tu1 present after shiftdown.

In contrast, *tuf3* transcripts appeared within 30 min of shiftdown, and reached a maximum after 1 h (Fig. 7c). The amount of *tuf3* transcript under these conditions was about ten times higher than that in transition-phase and stationary-phase cultures of J1681 (comparable amounts of RNA were used in each experiment, and autoradiogram exposure times were typically ten times longer for identification of the *tuf3* transcripts in J1681 than after nutritional shiftdown of M145). *tuf3* transcripts were barely detectable 3 h after shiftdown of M145.

The stringent response can also be elicited by treatment with SHX, although lower levels of ppGpp are induced than after nutritional shiftdown [75 and 200 pmol (mg dry weight)$^{-1}$, respectively; Fig. 8a]. While addition of SHX leads to a marked reduction in the level of rRNA transcripts (Strauch *et al.*, 1991), addition of SHX to a final concentration of 50 mM led to the appearance, within 30 min, of low-abundance *tuf3* transcripts (Fig. 8b).

DISCUSSION

tuf3 deletion mutants were not noticeably affected in growth or differentiation, demonstrating that EF-Tu3 does not play an essential role in the *Streptomyces* life-cycle. Western blotting using antibodies raised against the highly homologous *S. ramocissimus* EF-Tu3 failed to identify the *tuf3* gene product in total protein extracts isolated from M145 after nutritional shiftdown (van Wezel, 1994). This suggests that EF-Tu3, if present at all in *S. coelicolor*, occurs only in trace amounts, even when *tuf3* transcripts can be detected.

The XylE$^+$ phenotype of pISCT3-U1 revealed a promoter within 1900 bp of the *tuf3* translation start site. Earlier work (van Wezel, 1994) had shown that M145 containing pISCT3-2, which has only 260 bp of sequence upstream of *tuf3*, produces EF-Tu3 in the absence of any additional

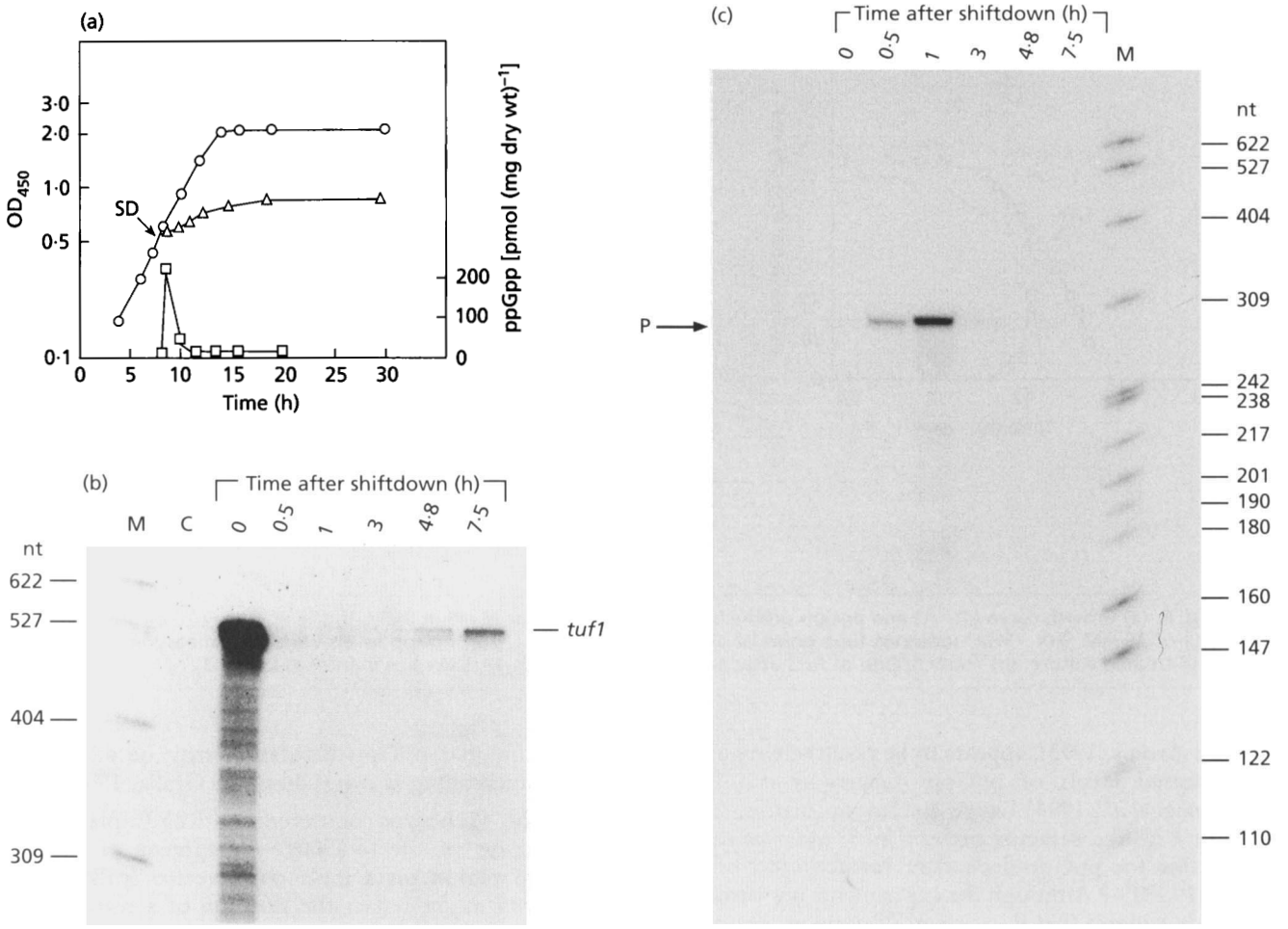

Fig. 7. Transcription of *tuf1* and *tuf3* after nutritional shiftdown of *S. coelicolor* M145. (a) Growth curve (△, ○) and ppGpp production (□) of a SMM-grown culture, with (△) and without (○) nutritional shiftdown (SD) at an OD_{450} of 0·6. (b) Transcription of *tuf1* after nutritional shiftdown. (c) Transcription of *tuf3* after shiftdown. P, *tuf3* transcripts initiated at the *tuf3* promoter around nt position −195; M, DNA size marker as in Fig. 3; C, control lane (30 μg tRNA).

promoter. Therefore it is possible that the strong *xylE* expression observed with pISCT3-U1 may be entirely due to a promoter located within 260 bp of the *tuf3* translation start site.

In vitro run-off transcription assays with *S. coelicolor* RNA polymerase revealed a transcription initiation site at approximately nt position −195 relative to the *tuf3* translation start site. The sequence of the putative *tuf3* promoter (TCGACG – 17 bp – GATGAT; Fig. 4) shows an 8 out of 12 nt match with the consensus sequences for the major class of eubacterial promoters (TTGACA – 17 bp – TATAAT; Hawley & McClure, 1983). The RNA polymerase fraction that yielded the highest level of *tuf3* transcription *in vitro* (Fig. 3) was enriched in RNA polymerase containing σ^{hrdB}, consistent with recognition by σ^{hrdB} *in vivo*. The *tuf3* promoter is preceded by a stretch of seven As and one T (Fig. 4), a very unusual sequence in the G+C-rich streptomycetes. Similar A+T-rich sequences found approximately 45 bp upstream of the transcription start site of various *E. coli* promoters were shown to interact with the α subunit of RNA polymerase, resulting in a stimulation of transcription (Ross *et al.*, 1993). The A+T-rich stretch near the *tuf3* promoter lies at a similar position, and it is possible that this sequence is involved in α-dependent transcriptional activation.

tuf3 transcripts appeared immediately after invoking the stringent response, either after nutritional shiftdown or after addition of SHX, suggesting that transcription of *tuf3* may be subject to positive stringent control, which may also be true for the histidine biosynthetic operon of *Salmonella typhimurium* (Rudd *et al.*, 1985; Cashel & Rudd, 1987). Another gene that may be subject to positive stringent control in *S. coelicolor* is *act*II-ORF4 (Fernández-Moreno *et al.*, 1991), the putative activator gene for the actinorhodin biosynthetic pathway (Takano & Bibb, 1994). However, in contrast to *tuf3*, *act*II-ORF4 transcripts can be detected in stationary-phase cultures of M145. Whether ppGpp plays a causal role in triggering the expression of these genes remains an open question. Interestingly, expression of *rpoS*, which encodes the stationary-phase-specific σ factor σ^S of *E. coli* (reviewed by

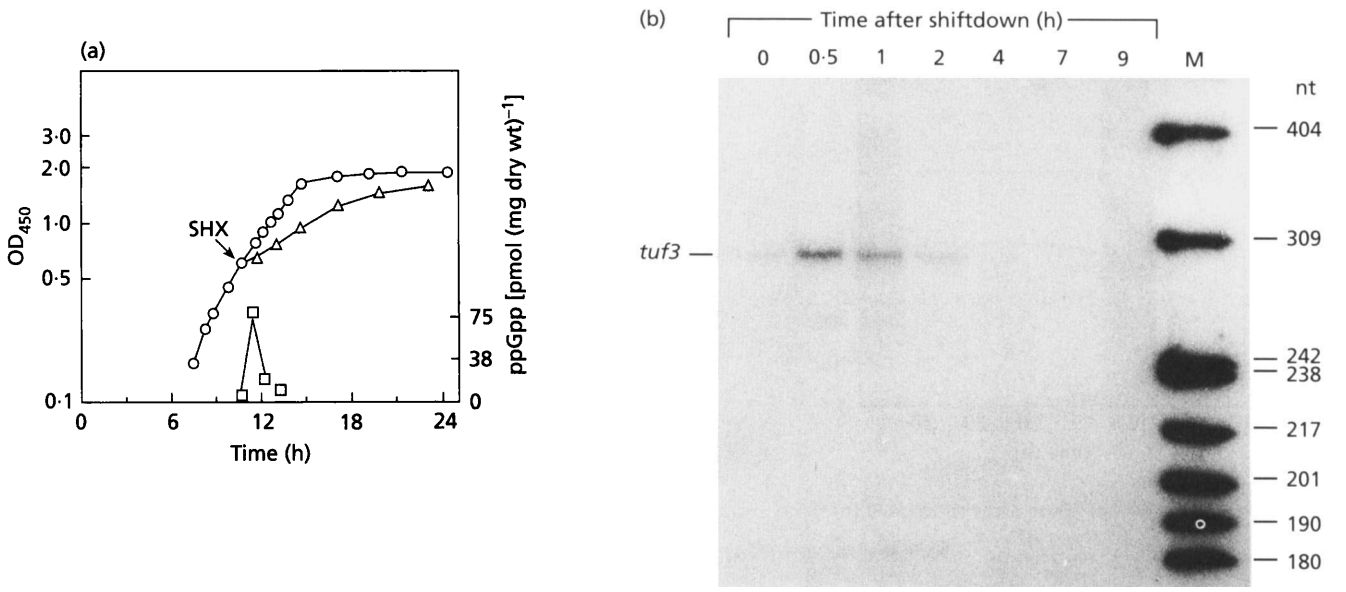

Fig. 8. (a) Growth curve (○, △) and ppGpp production (□) by *S. coelicolor* M145 in SMM, in the absence (○) or presence (△) of 25 mM SHX. 'SHX' indicates time point of addition of SHX (OD_{450} 0·6). ppGpp levels were only measured in the SHX-treated culture. (b) Transcription of *tuf3* after addition of 25 mM SHX. M, DNA size marker as in Fig. 3.

Hengge-Aronis, 1993), appears to be positively regulated by increased levels of ppGpp (Gentry *et al.*, 1993; Takayanagi *et al.*, 1994; Lange & Hengge-Aronis, 1994). Is there a σ^S-like σ factor present in *S. coelicolor* that is responsible for ppGpp-dependent transcription of *tuf3* and *act*II-ORF4? Although the experiments performed in this study suggest that P_{tuf3} is a σ^{hrdB}-type promoter, *E. coli* σ^S and σ^{70} (the major σ factor of *E. coli*) show overlapping promoter specificities (Tanaka *et al.*, 1993).

A low level of *tuf3* transcription was observed in late-exponential and stationary-phase cultures of the *bldA* deletion mutant J1681, but not in its *bldA*$^+$ progenitor J1501. If ppGpp does play a role in activating *tuf3* transcription, perhaps the sensitivity of the *tuf3* promoter to ppGpp is enhanced in the *bldA* mutant; alternatively, perhaps *bldA* mutants have elevated levels of ppGpp, reducing the incremental increase required to activate *tuf3* transcription.

While *tuf1* transcripts were abundant during exponential growth, roughly coinciding with transcription from the *rrnA* P1 and P2 promoters, transcription of *tuf3* was not observed in unstressed cultures. However, the *tuf3* upstream region gave a high level of *xylE* expression when cloned in a multi-copy promoter-probe plasmid, and high levels of EF-Tu3 had been observed previously in transformants containing either pISCT3-1 or pISCT3-2 (van Wezel, 1994). These multi-copy effects might reflect repression of *tuf3* by a protein that is readily titrated out when *tuf3* is present at high-copy-number. Alternatively, differences in the extent of DNA supercoiling of the chromosomally and plasmid-located *tuf3* promoters may (partly) explain the discrepancy in their activity: the degree of DNA supercoiling is known to influence the effect of ppGpp on transcription initiation *in vitro*, suggesting that ppGpp regulation may be affected by DNA supercoiling *in vivo* (Ohlsen & Gralla, 1992).

The bald phenotype observed on R2YE plates after introduction of the −1900/−5 segment of the *tuf3* upstream region on a multi-copy vector (pISCT3-U1) into M145 might reflect the titration of a transcription factor required for differentiation by a regulatory element contained in the cloned fragment. Other DNA fragments have been cloned that inhibit or prevent differentiation when present in *S. coelicolor* in multiple copies, most of which probably sequester proteins involved in development of aerial hyphae or spores (Champness & Chater, 1994). Examples are the putative σ^{whiG}-dependent promoters P_{TH4} and P_{TH270}, which probably titrate out this σ factor, leading to absence of sporulation (Tan & Chater, 1993). pISCT3-U1 overlaps pISCT3-1 by 500 bp at the *tuf3*-proximal end, and pISCT3-1 displayed normal growth and differentiation on R2YE; thus the putative regulatory element cannot be located near the *tuf3* promoter, and hence it seems unlikely that it is involved in regulation of *tuf3* transcription.

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